In Vivo ^{31}P NMR Studies of Corn Root Tissue and Its Uptake of Toxic Metals

ABSTRACT

Excised corn root tissue has been evaluated for its viability, integrity of compartmentation, intracellular pH gradients, total mobile phosphorus content and nucleotide concentrations under different levels of acidity, and mineral stresses using *in vivo* ^{31}P nuclear magnetic resonance spectroscopy at 21 to 23°C. Perfusion with Al^{3+} ion at low pH (4.0) for 20 hours caused the overall concentration of nucleotides in the cytoplasm to decrease significantly relative to the control. Respiratory activity as measured by O_2 uptake decreased by a comparable amount over this time period. The addition of glucose to the Al-containing perfusate negated the inhibitory effects on the respiratory system. Treatment of the tissue with paramagnetic manganese ion while perfusing in the presence of O_2 allowed for the observation of the sequence of events leading to the irreversible trapping of Mn^{2+} in the vacuole. Pretreatment of the roots with Mg^{2+} prevented Mn^{2+} migration to the vacuole over the time period of this experiment. Hypoxia prevented all but a limited uptake of Mn^{2+} into the cytoplasm of the root tips. No evidence of Mn^{2+} complexation of either cytoplasmic or vacuole Pi suggests that the energy derived from O_2 consuming processes is necessary for the facilitated movement of this divalent cation.

Both soil acidity and its effects on the liberation of toxic metals such as Al and Mn can produce adverse conditions for the growth and production of plants (4). Although much work has gone into the development of new strains of metal-tolerant plants to combat such problems, little is known concerning the physiological mechanisms responsible for stress tolerance or susceptibility in plants (3).

Low pH (<4.5) conditions in the soil can perturb the overall functioning of a plant and its ability to assimilate adequate levels of organic and inorganic nutrients. However, the exact effect of hydrogen ion concentrations on this phenomenon is often difficult to determine because of the occurrence of solubilized forms of toxic metals that are present under these conditions (3). With regard to the plant root system, it is thought that acid conditions are responsible for greater membrane permeability (4), impaired cation transport (19), metal displacement, and inhibition of metal uptake (9).

Al is among the most studied metals in terms of its toxic effects on plant function (5). Its specific action has been attributed to interactions with nucleotides, nucleic acids, membrane lipids, as well as important calcium regulating ATPase and phosphorylating enzymes (8). One consideration that mitigates against its intracellular action is its high charge and unlikely transport across the hydrophobic cell membrane. Some possible carriers that have been postulated to effect this process include negatively charged phospholipids, neutral complexes of citrate and Al^{3+}, as well as protein associated Al transporters formed by HII type phospholipid channels (8). In addition, the action of Al is known to be modulated by the presence of relatively high concentrations of divalent cations such as Ca and Mg (18, 24).

Another metal which poses a problem for plant development and production is divalent manganese. In general, Mn^{2+} is not as toxic to such a wide variety of plants as Al, yet its ease of transport across the cell membrane is well known (4). The presence of Mn within the plant has been shown to lower ATP concentrations and respiration rates as well as alter the activity of enzymes and hormones (32). In some instances healthy roots have been shown to reduce Mn^{2+} toxicity by precipitating oxidized Mn as MnO_2 on root surfaces (4). Plants such as maize, however, may avoid Mn^{2+} toxicity by entrapment of relatively high concentrations of the metal in nonmetabolic centers such as the vacuole (2). A similar observation has also been suggested for the sequestration of toxic levels of Cu^{2+} (37).

^{31}P NMR has been used extensively for *in vivo* experiments to evaluate the intracellular pH as well as identify and determine the level of mobile phosphorus compounds contained in both animal and plant tissues (6, 25, 28). Recently, reports of ^{31}P NMR studies of various plant tissue (14, 15, 25), seeds (15, 20), and leaves (36) have appeared. These studies have clearly demonstrated that this technique can be exploited to measure the energy status, (ATP/ADP ratios) (25), changes in intracellular pH with binding of uncouplers of oxidative phosphorylation (12), phosphorylation of different monosaccharides (12), effects of hypoxia on pH regulation (26), effects of sodium on phosphate uptake (29), Mn entrapment (12), and the rate of mitochondrial synthesis of ATP under different conditions via saturation transfer (30).

In this report, we present results of 161.7 MHz ^{31}P NMR studies of corn root tip tissue in which we have examined the effects of an acid environment as well as the effect of glucose and toxic metal ions such as Al and Mn on overall viability, as manifested in the level of respiration, ATP, and maintenance of intracellular pH. In addition, we demonstrate the effect of hypoxia on the transport of Mn^{2+} across the cell membrane.

MATERIALS AND METHODS

NMR Experiments. Approximately 700 to 1000 corn root tips (*Zea mays* L. var FRB-73) were excised from seedlings grown at 28°C in a growth chamber over damp paper for 72 h as described earlier (34). Immediately after cutting, the 5 to 7 mm tips were placed in a cold neutral solution containing (30 ml) 0.1 mM $CaSO_4$ and aerated with O_2 for a period of approximately 0.5 h. When working in the pH 4.0 range the roots were allowed to remain in the O_2 aerated solution for 4 h and washed thoroughly prior to examination by NMR. The tips were then transferred to a 10 mm NMR tube equipped with inlet and outlet perfusion

tubes similar to those described by Lee and Ratcliffe (14). However, we have found that faster perfusion rates (~45 ml/min) could be achieved if a single, wider (2 mm) exit tube at the lower end of the NMR tube was used since the glass wool at the exit tended to restrict the flow and cause overflowing at the faster flow rates with narrower tubing. This faster perfusion rate gave better reproducibility and sharper spectra than obtained with rates of 10 to 20 ml/min. The perfusion medium (1000 ml) was buffered to pH 6.0 with 10 mM Mes buffer (pH 7.5) with bis tris propane, or not buffered at a specified pH as described. Unless otherwise indicated, each solution contained 50 mM glucose, 0.1 mM $CaSO_4$ in addition to the specified concentration of metal ion being used. In hypoxia experiments, N_2 was substituted for O_2 as the saturating gas that was bubbled into the reservoir containing approximately 500 to 1000 ml of perfusate. To change the perfusion medium, a second reservoir was interconnected by means of a three-way stopcock assembly, and the intermediate tubing between was primed with the new perfusate prior to switchover. Upon switching to the new perfusate, 80 ml of the returning perfusate was discarded (approximate volume of the connecting tubing and NMR tube) to flush the system.

The 161.7 MHz ^{31}P NMR spectra (obtained with a 54 mm narrow bore spectrometer) were accumulated at 21° to 23° over a 16,000 Hz frequency range with 2,000 data points zero filled to 16,000. Each rapidly acquired spectrum required 10,000 to 40,000 transients with a repetition time of 0.162 s (total time of accumulation 27 to 108 min unless indicated otherwise), utilizing approximately a 30° pulse (12 μs) without broadband decoupling. Spectra used for making quantitative evaluations required 16 s pulse delays and 35 μs 90° pulses. Spectra were obtained consecutively, and stacked sequentially onto the disk memory. Following the completion of the experimental time course, each spectrum was normalized (S/N) (allowances made for computer scaling) to the initial spectrum for comparison of relative concentrations of mobile phosphorus compounds. The relative area changes in each experiment were determined from the average of three independent experiments and three control experiments in which the perturbing species was omitted. The standard deviation for the observed changes in area for each set of experiments was between 8 and 10%. Each shift position area was normalized to the area of the external reference resonance (0.120 M) HMPA[1,2] kept in a sealed capillary in the center of the tube.

The concentrations of mobile phosphorous compounds in the root tissue sample, (average over the total sample volume within the detector) were determined by comparing the area of the signal from the HMPA capillary reference (used in the tissue spectra) with its area relative to the area of the phosphorus resonances obtained from 1 mM standard solutions of ATP, glucose-6-P and Pi. Both the tissue spectra and standard solution spectra were acquired under quantitative conditions. Adjustments for differences in signal responses were applied to establish a direct relationship between the areas of the resonances obtained in the slowly and rapidly acquired spectra. Plots of pH *versus* ^{31}P chemical shifts were made for Pi with reference to external MDP as described previously (29). The values for these shifts were adjusted for the chemical shift difference between the external reference (0.120 m) HMPA, 13.78 ppm and MDP, 0.00 ppm. Using the value of 13.78 ppm for HMPA, our pH-dependent shifts were determined on a scale consistent with those reported by Roberts *et al.* (27). The advantage of using HMPA throughout these studies are 2-fold: (a) It shows no pH dependence as does MDP. (b) Unlike MDP, HMPA is stable for indefinite periods of time over all of the temperature ranges studied.

Spin lattice relaxation (T_1 measurements were made by the inversion-recovery method 180°-τ-90° with a 12 s repetition time for the non-nucleotide resonances and 4 s for the nucleotide resonances between scans. Relaxation values were calculated using a two-parameter exponential fit.

Estimates of intracellular pH were made using the standard reference curve for Pi suggested by Roberts *et al.* (31), 5.0 mM K_2HPO_4, 2.0 mM $MgCl_2$, and 100.0 mM KCl.

Respiration Measurements. Approximately 100 corn root tips (3–5 mm in length) were placed in a solution at 25°C under constant aeration for 2 h. The O_2 consumption rate was then measured polarographically with a YSI model 532 oxygen meter[3] by stopping the aeration momentarily. The measurement was repeated after 20 h of aeration in the same incubation medium.

RESULTS AND DISCUSSION

Spectral Characteristics—^{31}P Relaxation and Chemical Shifts. A representative 161.7 MHz ^{31}P NMR spectrum of 5 to 7 mm corn root tips taken under the fast acquisition conditions (Experimental) is given in Figure 1A (see Fig. 1 legend for shift assignments). Since this spectrum was acquired using a 30° pulse angle and fast repetition rate, the area of the sugar phosphate and Pi resonances (downfield group −12 to −16 ppm)/nucleo-

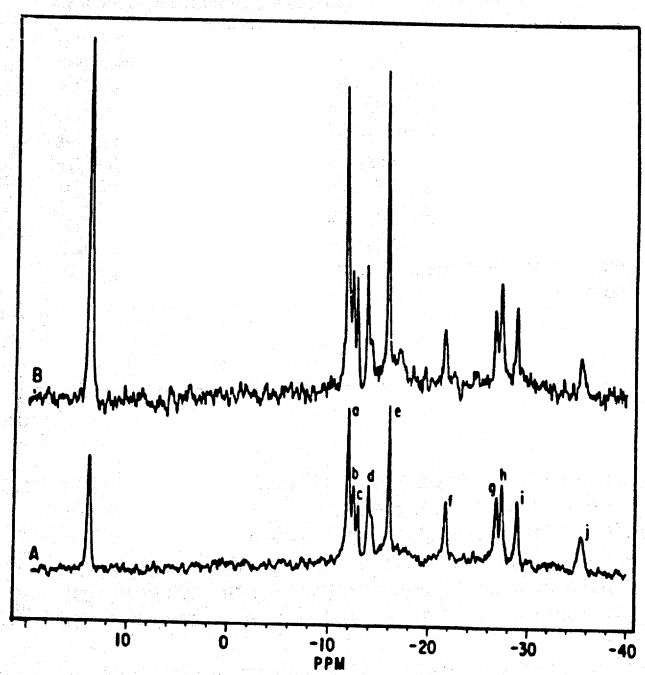

FIG. 1. A, 161.7 MHz ^{31}P spectrum of approximately 900 excised root tips (5–7 mm) taken under fast acquisition accumulation parameters, 30° pulse (12 μs) 2,000 data points zero filled to 16,000 recycling time of 0.162 s, 10,000 transients, frequency of 16 KHz, 15 Hz line broadening, total time for acquisition of spectrum 27 min. B, Slow recycling time full 90° pulse generated spectrum obtained with a 16-s recycling time, 1,000 scans, 16,000 Hz frequency, 15 Hz linebroadening, total time for acquisition of spectrum 3.5 h. The assignments of resonances are as previously described (27): a, glucose-6-P; d, cytoplasmic Pi; e, vacuole Pi; f, γATP; g, αATP; h, i, UDPG and NAD nucleotides; j, β-ATP. Resonance b and c are thought to be fructose-6-P and AMP, respectively. See discussion on hypoxia and glucose deprivation at low pH.

[1] Abbreviations: HMPA, hexamethylphosphoramide; MDP, methylenediphosphonic acid.

[2] Caution should be exercised in handling this chemical since it is a known carcinogen.

[3] Reference to brand or firm name does not constitute endorsement by the United States Department of Agriculture over others of a similar nature not mentioned.

tides (upfield group −21 to −35 ppm) is highly distorted, *i.e.* the former group has T_1 values in the 2.7 to 3.5 s range, whereas the nucleotide resonances have T_1's of less than 1 s (see Table I). The vacuolar Pi resonance has longer T_1 value than the cytoplasmic Pi, presumably a consequence of the greater viscosity inherent in the cytoplasm.

The change in the relative concentration of each mobile phosphorus compound as determined by its resonance area could only be compared with those in the same range of relaxation when we employed rapid recycling times, *i.e.* those resonances with short relaxation times of 300 to 800 ms (ATP, UDPG, and NAD nucleotides) were compared to each other while the resonances in the 2.7 to 3.5 s relaxation domains, *i.e.* sugar phosphate and Pi, were only measured against each other. The total concentrations of mobile phosphorus were compared from run to run by cutting out and weighing the entire normalized spectra.

Other sources of phosphorous such as immobilized phospholipids, nucleic acids, and bound forms of PPi give no sharp ^{31}P signal (a slight rise in the spectral baseline may result from these relatively immobile species) in these experiments (25).

Using the fast acquisition regime, we effectively suppress the observed spectral responses associated with the resonances in the downfield area of the spectrum. The true differences in the concentration represented by the two domains of these spectra is evident in the spectrum shown in Figure 1B in which a full 90° pulse and 16 s repetition time were used to obtain spectra. Because these spectra (Fig. 1, A and B) have been normalized to the fastest relaxing component, the ATP resonances, we can clearly see the gross underestimation (52% loss) of the sugar phosphate and Pi signals in Figure 1A. The undistorted spectrum (Fig. 1B) reveals that the nucleotide concentrations represent approximately 32% of the total mobile phosphorus in the root tips. This corresponds to a value of 15 mol %. Roberts *et al.* (29) have recently reported a value of 7.5%. Presently we have no reasonable explanation for this disparity.

As in previous studies (15), we have used the fast acquisition time spectra throughout our work to characterize the relative changes taking place within the high and low field regions of the spectrum. Obviously, changes in concentration of one species can result in changes in the concentration of another species (*e.g.* hydrolysis of ATP to Pi_{cyt}). However, because of the extreme disparity in T_1's and concentration levels, even a large decrease in ATP concentration, as examined in the fast acquisition spectrum, for example, would only yield a correspondingly small response in the ^{31}P resonance area of an equivalent amount of liberated Pi. Any noticeable changes in the areas of the low field long T_1 resonances corresponds to a larger real percentage change in total of mobile phosphorus when examined using this rapid acquisition condition for obtaining spectra. However, differences seen in the area of the high field nucleotide resonances (*i.e.* ATP, UDPG, and NAD) reflect little change in the overall mobile phosphorus concentration. Note that the linewidth of vacuolar Pi in our spectra are considerably narrower ($\delta^{1/2}$ of 40 Hz) when compared to those greater than 100 Hz reported in previous studies of maize root tips (30). In addition, this resonance is less intense, representing only 1.5 mM phosphorus compared to 9.0 mM as reported earlier (29). It is likely that the broader vacuole Pi resonance seen in earlier studies is a consequence of paramagnetic metal contamination within the vacuole due to accumulation from the soil in which the corn was grown, since line narrowing due to lengthening of T_1 was affected with sequestering agents. Our overall lower vacuole Pi concentration may in part be a consequence of cultivar, size or age differences. The concentrations of glucose-6-P and cytoplasmic Pi were 1.3 and 0.7 mM, respectively, in this sample. Concentrations as high as 0.7 mM for ATP have been observed in our samples from time to time.

In general, the pH values obtained for cytoplasmic Pi in our studies are consistently 0.2 to 0.3 pH units higher than those previously reported (14). We suggest that these slightly higher values are possibly due to the faster perfusion rates (45 ml/min) used in our experiments. Also, our shift range of Pi (Fig. 2) from pH 4.5 to 7.8 is −16.02 to −13.80 ppm. The previously reported shift region given for this pH range was −16.50 to −14.20 ppm (27). Our range of shifts correspond to those reported earlier by Garlick *et al.* (7). The difference in range of shifts may be a function of bulk magnetic susceptibility contributions with different magnet and sample geometries (17). Under optimum conditions, pH 6.0, 50 mM glucose, 0.1 mM $CaSO_4$, O_2, and rapid perfusion 45 ml/min, we observe a cytoplasmic Pi shift of −13.94 and a vacuole Pi shift of −16.00, corresponding to intracellular pH values of 7.6 and <5.5, respectively (see pH profile, Fig. 2). A comparable pH curve was obtained when MDP was used as a reference. These pH conditions could be maintained for longer than 24 h with no noticeable change in total phosphorus concentrations or its distribution among the mobile components observed in the spectra. Comparison of chemical shifts with standards suggest that the resonances at −12.60 ppm and −13.00 ppm at an intracellular pH of 7.5 tentatively correspond to fructose-6-P (f-6-P) and partial contribution from AMP, respectively.

Stability of Root Tissue. To accurately evaluate the long-term effects of various stress factors on the status of excised metabolizing root tissue on our NMR experiments, we undertook an extensive study to determine basal stabilities of this root tissue over extended periods. In general, the spectra of excised root tips

Table I. *^{31}P NMR Spectral Characteristics of Intracellular Components of Maize Root Tips*

Compound	T_1	δ ppm[a]	pH
	ms		
G-6-P	2.89 ± 0.24 (<3.0)[b]	−11.94	
Pi vacuolar	3.58 ± 0.5 (<2.0)[b]	−16.00	<5.5
Pi cytoplasmic	2.78 ± 0.31 (1.63)[c]	−13.96	7.6
ATP			
γ	0.35 ± 0.06 (0.37)[c]	−21.76	
α	0.39 ± 0.05	−26.76	
β	0.21 ± 0.02	−35.46	
UDPG	0.63 ± 0.06	−27.31 −28.85	

[a] Chemical shift of HMPA, assigned a value of 13.78 ppm relative to MDP (0.00 ppm). [b] Roberts *et al.* (29). [c] Roberts *et al.* (30).

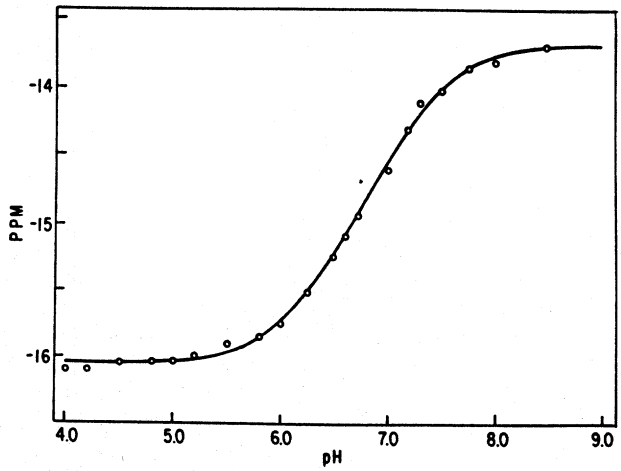

FIG. 2. pH *versus* ^{31}P chemical shift at 22°C. Solution contained 5.0 mM K_2HPO_4, 2.0 mM $MgCl_2$, and 100 mM KCl. Shifts are referenced to 0.120 M HMPA in a centrally located capillary. The shift position of HMPA was assigned a value of 13.78 for consistency with the previously reported MDP reference scale (13).

show essentially no change in total area (concentration of mobile phosphorus compounds) or relative changes in concentrations of these components for periods up to 23 h when perfused with a buffered medium containing 0.1 mM $CaSO_4$ at pH 7.5. Addition of 50 mM glucose into the perfusion medium does not affect the overall stability of the spectra. However, up to 20% increase in the concentration of glucose-6-P and 10% increase in the concentration of ATP was noted over a 20-h period (spectra not shown). Intracellular compartmentation and initial pH values of these compartments could be easily maintained for up to 73 h. However, a decided increase in the vacuolar Pi resonance area resulting in an increase in the total amount of observed mobile phosphorous concentration of ~20% became apparent after this time period.

Perfusion of the root tissue with a medium containing 0.1 mM $CaSO_4$ at a pH of 3.5 appeared to destroy cellular integrity within 20 h, giving rise to significant phosphorus leakage and a loss of approximately 40% of the mobile phosphorus compounds as evidenced by the ^{31}P spectrum (data not shown). A pH of 4.0, on the other hand, does not appear to cause cellular breakdown in this time period. However, as shown in Figure 3, a significant increase (after accounting for liberated phosphate from nucleotides) of 35% of total non-nucleotide observable signal (major increment in vacuole Pi) is seen after 20 h of exposure in this acid environment. It is significant that this added signal area corresponds predominantly to transferred Pi to the vacuole. This effect is not evident over a period of 20 h when the perfusate surrounding the roots is kept at a relatively neutral pH (see above). It is presumed that this additional signal is generated by the mobilization of phosphate derived from the liberation of bound phosphate trapped in the cell walls since the 12% loss of signal from ATP and 13% loss of UDPG signal cannot contribute significantly to this increased area. Further evidence to support this contention is seen in experiments in which the root tips were extensively washed and suspended in 0.1 mm $CaSO_4$ solution for four or more hours prior to performing the NMR experiments. In these studies less than 20% phosphate accumulation was observed in the vacuole. It is not clear why the phosphate is preferentially moved into the vacuole. However, one might speculate that under the low pH conditions surrounding the plasmalemma, the necessity for using ATP for proton pumping to the exterior is severely diminished, and therefore the ATP concentration remains relatively constant. In turn, the excess Pi

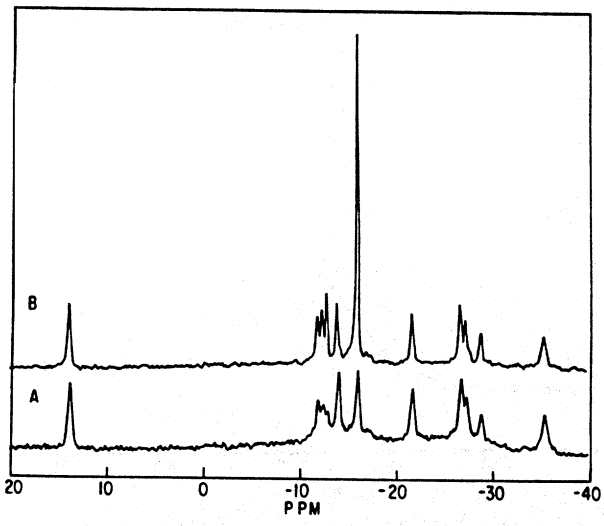

FIG. 3. A, 161.7 MHz ^{31}P spectrum of approximately 900 excised root tips (5–7 min) taken under the fast acquisition conditions following 2 h in the NMR perfusion system, pH = 4.0, 0.1 mM $CaSO_4$, 40,000 transients. B, Same as (A) after 20 h of perfusion.

that invades the cytoplasm may exceed that which is necessary to maintain a satisfactory cytoplasmic phosphorylation potential (16) given by:

$$\frac{[ATP]}{[ADP][Pi]}$$

leading to the rapid migration of phosphate to the vacuole. A similar preferential uptake of Pi in the vacuole under acid conditions has been noted earlier (12, 27). During this period, there is a significant depletion (20%) of glucose-6-P (a). The undetermined resonance at −13.00 ppm (c), which we suspect has some contribution from AMP, has essentially doubled in concentration. Based on the depletion of glucose-6-P and UDPG (a, h, and i) along with a relatively small reduction, (12%) in ATP concentration, we speculate that adenylate kinase activity has provided an alternate pathway to the production of ATP (see legend of Fig. 1 for assignments). The corresponding increase in the resonance at −13.00 ppm could be attributable to the under utilized AMP produced (Fig. 3). An increase in the same resonance (−13.00 ppm) is also noted in our spectra of this tissue stressed with hypoxia (26). However, quantitation of this increase under such conditions is difficult because of the severe peak overlaps in this region at low intracellular pH. More details about the chemistry of the hypoxic state in connection with metal transport will be discussed later. A similar experiment conducted with perfusion of 50 mM glucose at pH 4.0 showed essentially no change in ATP concentration, no depletion of glucose-6-P or UDPG or increase in the −13.00 ppm resonance after 20 h of perfusion. Since ATP and glucose-6-P levels are maintained at high rates of respiration (30) during this period with excess glucose, no additional production of AMP would be expected. As we observed in the experiment without glucose, up to 40% additional phosphorus signal was generated during this 20 h period. No change in intracellular pH was noted throughout this experiment, *i.e.* the cytoplasm was steady at pH 7.5 and the vacuole remained below 5.5 within the limits of its measurement.

Effects of Aluminum on Root Tissue Viability. To evaluate the toxic effects of Al on root tissue, it was essential that we study these effects at a pH between 4 and 5 since Al becomes mobile in the soil in this pH range (3). Furthermore, since it is presumed that the active transported species associated with the toxicity is Al^{3+}, it is important to work in a pH region that favors the formation of this ion. At a pH of 4.0 and pAL of 4.0 ($-\log [Al]_{total}$), the speciation of Al is approximately 47% Al^{3+}, 47% $Al_7(OH)_{17}^{4+}$, and 6% $Al(OH)^{2+}$ (21). Although we could effectively increase Al^{3+} concentrations to nearly 95% (21) by working at pH 3.5, this highly acid environment as indicated above causes excessive membrane degradation over the 20 h duration of these experiments. Thus, we examined the effects of Al at pH 4.0 in unbuffered solution containing 0.1 mM $CaSO_4$ with and without glucose. Table II summarizes the effects of the perfusion experiments at pH 4 in the presence of Al, Al and glucose, and the absence of both on the status of maize root tips after 20 h. Figure 4 shows the results of this treatment after 20 h of exposure to the perfusate containing 0.1 mM Al and 0.1 mM $CaSO_4$. Unlike the control experiments conducted at pH 4.0, the Al^{3+} infiltration as seen in Figure 4 clearly diminished the level of ATP by 65% based on comparison of the γ and α ATP resonance areas. This loss of signal might be attributed to broadening brought about through complexation. However, such complexation is not significant until pH 4.0 (the binding constant of Al to ATP is one order of magnitude smaller than Mg at pH 7.5) (10). The UDPG levels have diminished by 65% as well. Levels of all other nonnucleotide species (except glucose-6-P which dropped an additional 20 ± 2.6%) have not changed when compared to the control experiment without Al (Fig. 3) within experimental error.

If we take account of the loss of the nucleotide phosphate area

Table II. *Effects of pH, Aluminum, and Glucose on the Nucleotide and Total Mobile Phosphorus in Maize Root Tips After 20 h Treatment*

Condition	Change in Phosphorus Signal Area[a]	Change in ATP[b]	Change in UDPG[b]	O_2^c Consumption Rate
		%		
pH 4.0, 0.1 mM $CaSO_4$	+35	−12	−13	1.15
pH 4.0, 0.1 mM $CaSO_4$ 50 mM Glucose	+40	0	+5	1.10
pH 4.0, 0.1 mM $CaSO_4$ 5×10^{-5} M $Al_2(SO_4)_3$	0	−65	−65	0.20
pH 4.0, 0.1 mM $CaSO_4$ 2.5×10^{-3} M $Al_2(SO_4)_3$	+20	-45	−50	0.40
pH 4.0, 0.1 mM $CaSO_4$ 50 mM Glucose 2.5×10^{-3} M $Al_2(SO_4)_3$	−10	−5	−10	0.90

[a] Area of non-nucleotide phosphorus after accounting for contribution from nucleotide breakdown. [b] All values ± 10%. [c] Relative respiration rates at 20 h as compared to 2 h as measured by O_2 tension at 25° (see "NMR Experiments").

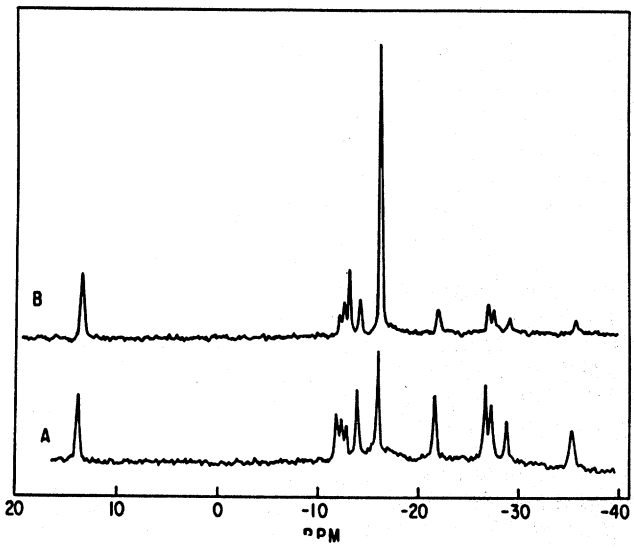

FIG. 4. A, ^{31}P spectrum (40,000 transients) taken as above after 4 h of perfusion with a solution of 0.1 mM $CaSO_4$ (pH 4.0) O_2. B, Same perfusate containing 50 μM $Al_2(SO_4)_3$ (pH 4.0) after 20 h of exposure.

which corresponds to the generated Pi signal, we note that essentially no additional ^{31}P signal area is observed in the 20 h spectrum. This probably means that additional phosphate compounds have precipitated with migrating Al in the cell wall before reaching the interior of the cells. In contrast, up to an additional 40% of predominantly Pi signal is seen after 20 h in the absence of Al (Fig. 3).

When the concentration of Al in the perfusate was raised to 5 mM, only a 45% loss of ATP and a 60% drop in respiration was noted. These observations suggest that the predominance of the polymeric hydrated forms of Al present at such high concentrations of Al preclude the movement of Al^{3+} across the plasma membrane. Also, less aluminum phosphate precipitation has taken place since the overall phosphate signal has increased by 20% (see Table II).

It is presumed that the action of Al is exhibited most dramatically on mitochondrial activity as suggested by the significant drop in ATP levels. This drop is supported by the observed inhibition of respiration levels seen in Table II. A similar inhibition of respiration has been noted by Norton (22) on sanfoin roots after exposure to Al. In addition, other phosphorylation processes may also be affected since glucose-6-P and UDPG intensities have also fallen. The former observation concerning glucose-6-P concentrations may be a significant finding since it has already been shown experimentally (33) and clinically (1) that Al can inhibit the activity of hexokinase. The depletion of UDPG can be tied to the phenomenon of limited growth and stunting of roots exposed to Al-rich soils (3) whereby the suppression of this glucose carrier limits the production of cell wall polysaccharides.

Although plant exposure to Al over its growth period is significantly longer than the time course of these experiments, we believe that our observations point to the disturbance of some important primary biochemical events that ultimately result in an overall diminution in plant production. It is interesting to note that Wagatsuma (35) attributed the enhanced Al uptake under a N_2 atmosphere to an increased membrane permeability. Alternatively, their results may simply be a consequence of a more pronounced absorption of Al to the highly charged cell walls induced by the decrease of the transplasma membrane proton pumping activity under nitrogen. Thus, the excessive Al accumulation may reflect cell wall entrapment rather than cell penetration.

The experimental results shown in Figure 5 indicate that the presence of glucose appears to nullify the detrimental effect shown by Al without glucose as depicted in Figure 4. From the relatively constant concentration of ATP as well as respiration levels observed over the 20 h period, it appears that the combination of glucose and Al has little or no effect on mitochondrial activity (Table II). There appears to be a small loss of the total phosphorus signal (−10%) characterized by a significant depletion of vacuole Pi (−68%) and a modest 11% increase in cytoplasmic Pi. From the small upfield chemical shifts of ~0.15 ppm on the γ and β ATP resonances and the observed broadening of the β resonance seen after 20 h of exposure to the Al and glucose perfusate, we might speculate that Al does in fact penetrate the plasma membrane, however its presence in the cytoplasm is barely detectable. Nevertheless, the constant supply of glucose in the medium may provide additional energy to efficiently transport Al through the tonoplast where it can effectively be complexed with Pi at pH 5.5 or below. Thus, the overall loss of the phosphate signal may be a consequence of precipitation or aggregation of aluminum phosphate in the vacuole (10). Because of the enhanced movement of Al into the vacuole, levels of cytoplasmic Al are never allowed to accumulate to concentrations which can seriously impair the mitochondrial activity.

Uptake and Intracellular Movement of Manganese in Root Tip Cells Under Aerobic and Hypoxic Conditions. Although the

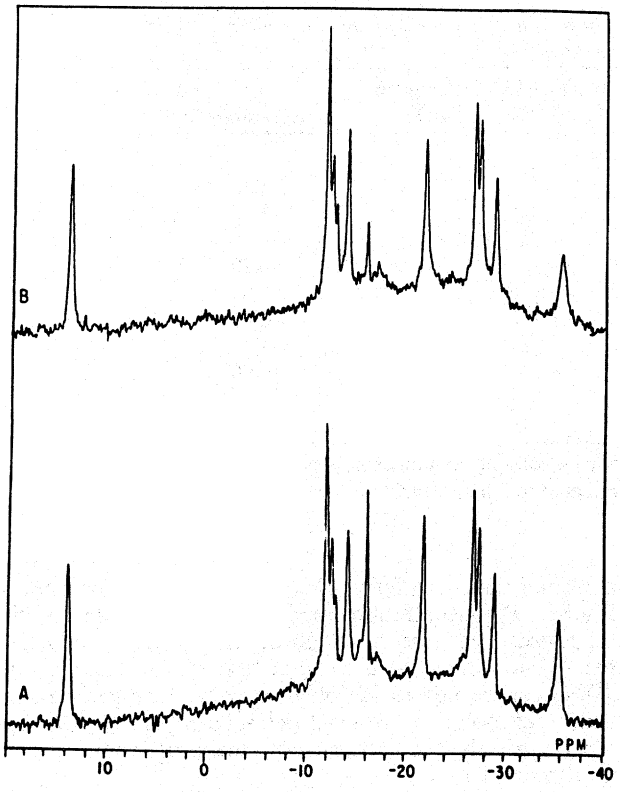

FIG. 5. A, ^{31}P spectrum (40,000 transients) taken as above after 4 h of perfusion with a solution of 0.1 mM $CaSO_4$, 50 mM glucose (pH 4.0) O_2. B, Same perfusate containing 2.5 mM $Al_2(SO_4)_3$ (pH 4.0) after 20 h of exposure.

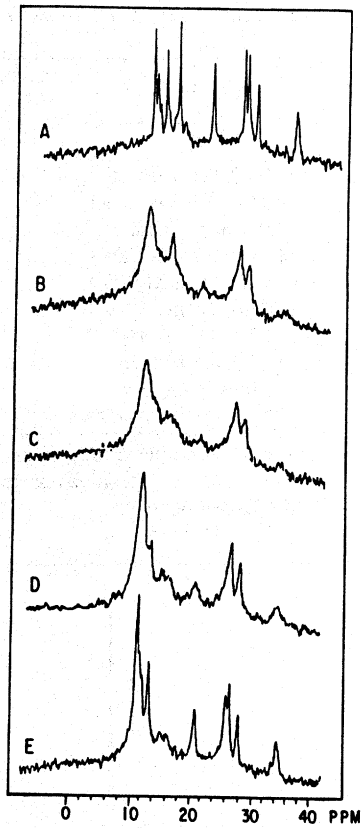

FIG. 6. A, ^{31}P spectrum (10,000 transients) taken as above after 3 h of perfusion with a solution of 0.1 mM $CaSO_4$, 50 mM glucose, 10 mM Mes buffer (pH 6.0) O_2. B, Same as above except for the addition of 1.0 mM $MnCl_2$ and exposure for 54 to 81 min. C, Same as (B), exposure 108 of 135 min. D, Sample perfused with buffer as in (A) for 0 to 27 min. E, Same as (D) for 81 to 108 min.

mechanism for movement of divalent ions into plant cells is not well established, it is presumed that it requires either a facilitated carrier or energy-dependent channeling process as elucidated in animal tissue (23).

Previous ^{31}P studies (12) have demonstrated that Mn^{2+} does enter the maize root cell as evidenced by significant resonance linebroadening. However, because of the limited spectral resolution, it was difficult to ascertain the sequence of events in this process throughout the Mn^{2+} influx and efflux. We have reexamined this experiment in somewhat greater detail in order to begin to understand the mechanism by which divalent metal ions such as Mn are transported to various compartments within the cells.

Figure 6 depicts a sequence of ^{31}P spectra of root tips perfused under aerobic conditions with 50 mM glucose, 0.1 mM $CaSO_4$, buffered to pH 6.0 with 10 mM Mes (Fig. 6A) and tips subsequently exposed to 1.0 mM $MnCl_2$ in the same buffered medium for a period of 135 min (Fig. 6, B and C). During the first 54 min, Mn^{2+} affects the ATP signals as evidenced by the paramagnetic broadening of the γ, α, and β resonances (not shown). This phenomenon, as described previously (12) is to be expected, since ATP has a stronger binding constant for Mn^{2+} when compared to those of the other mobile phosphorus compounds in the cytoplasm. In addition, Mn^{2+} can easily displace Mg^{2+} from ATP since Mn^{2+} binds ATP four times more strongly than Mg^{2+} (11). Within 54 to 81 min (Fig. 6B), the resonance at -13.96 ppm representing cytoplasmic Pi has also undergone paramagnetic broadening, signifying that Mn^{2+} has saturated ATP and is complexing which other phosphate components in the cytoplasm. Final migration of Mn into the vacuole is not observed until 108 to 135 min in which paramagnetic broadening of vacuole Pi at -16.06 ppm is complete (Fig. 6C). After 0 to 27 min of perfusion with non-Mg containing buffer, the reversible movement of Mn^{2+} across the plasma membrane is observed by the reappearance of the ATP and cytoplasmic Pi resonances (Fig. 6D). After 81 to 108 min of this treatment (Fig. 6E), the original spectrum minus the resonance representing vacuolar Pi has been restored. Respiration rates for this tissue before and after Mn treatment were the same. Further washing for up to 5 h failed to move the paramagnetic Mn^{2+} back across the tonoplast membrane. We note that traces of Mn^{2+} are probably still present in the tissue as evidenced by the generally broadened appearance of the spectrum relative to the spectrum of the initially untreated tissue. Furthermore, we observe that the resonances representing the sugar phosphates have increased in size by almost a factor of 2. This is due either to a shortening of the relatively long T_1's associated with the sugar phosphates because of traces of Mn^{2+} (*i.e.* artifactual increase), or to a stimulatory effect of Mn^{2+} on the uptake of glucose and subsequent ATP-hexokinase mediated phosphorylation (*i.e.* true increase in G-6-P). The same experiments as above performed in the absence of glucose in the perfusate showed little or no change in the intensity of the glucose-6-P peak following the washing out of Mn^{2+} with buffer. Thus, the latter explanation may have some merit. Moreover, we observed that without glucose, movement of Mn^{2+} (as evidenced by the broadening of the Pi signal) into the vacuole takes 30% longer than when glucose is present. This finding suggests that the energy required to facilitate the movement of Mn^{2+} across the tonoplast is enhanced by a constant supply of available glucose. It is important to note that the presence of Mn^{2+} has had no significant effect on the maintenance of intracellular pH or respiration within the time course of these treatments nor are there any significant losses in nucleotide concentrations observed

following 5 h of Mn^{2+} treatment.

Preincubation of the root tips with the same buffer solution containing 1.0 mM $MgCl_2$ gave somewhat different results from the above when the tissue was subsequently exposed to 1.0 mM $MnCl_2$. Figure 7 shows that after 108 to 135 min of Mn^{2+} treatment of the pretreated tissue, both Pi resonances persist (Fig. 7D). Washing of the tissue with buffer alone after 18 h (Fig. 7E) restores the nucleotide to a somewhat lower level with little change in the concentration of all other components. These results indicate that the metal carrier within the membrane or other binding sites within the cell can be saturated with Mg^{2+} prior to the treatment with Mn^{2+}. Such treatment has the effect of suppressing the binding and subsequent broadening effects of Mn^{2+} in the cytoplasm and the vacuole.

To get a clearer understanding of the mechanism responsible for the movement of divalent metals into the cell, we attempted to study the uptake of Mn^{2+} into the root tissue under an atmosphere of N_2 (hypoxic state). As described previously by Roberts *et al.* (26), hypoxia is manifested in the ^{31}P spectra by a lowering of the cytoplasmic pH (lactic acid production), a 40 to 60% loss of ATP, UDPG, sugar phosphates, and a large increase in cytoplasmic Pi. In addition, the generation of small amounts of ADP is also observed, seen as a small shoulder at −22.90 ppm.

Comparison of spectrum Figure 8A (aerobic) with Figure 8B (hypoxic) shows the full effect of a 54 to 81 min exposure of the root tissue to the nitrogen saturated perfusate. The cytoplasmic Pi resonance has shifted from −13.96 (pH 7.6) to −14.38 ppm (pH 7.0), cytoplasmic Pi has increased dramatically in intensity while glucose-6-P and ATP have been depleted. Under hypoxia, treatment of the roots with 1.0 mM $MnCl_2$ results only in a broadening of the already weak ATP resonances after 108 to 135 min (Fig. 8C). No indication of additional broadening of any other resonances in the spectrum are apparent. Attempted washing out of the bound Mn for up to 108 min under the nitrogen atmosphere was unsuccessful as seen in Figure 8D. However, reintroduction of O_2 into the system activated the migration of the low level of Mn^{2+} back out across the plasma membrane and regenerated the sharp narrow resonance spectrum as seen previously (Fig. 8E).

The results of this experiment are significant in terms of their impact on our understanding of the energetics responsible for the movement of divalent metals into plant cells. During a state

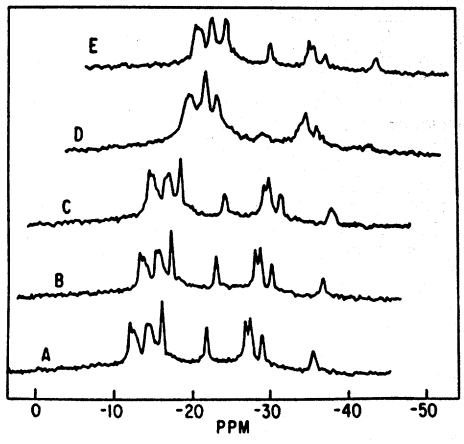

FIG. 7. A, ^{31}P spectrum (10,000 transients) taken as above perfused with a solution containing (A) 0.1 mM $CaSO_4$, 50.0 mM glucose, 10.0 mM Mes buffer (pH 6.0) O_2, and 1.0 mM $MgCl_2$ for 0 to 27 min. B, Same as (A), 27 to 54 min. C, Perfusate in (B) washed out with buffer and changed to buffered solution containing 1.0 mM $MnCl_2$, 0 to 27 min. D, 108 to 135 min period of treatment with 1.0 mM $MnCl_2$ solution. E, Spectrum after 18 h of washing with buffer.

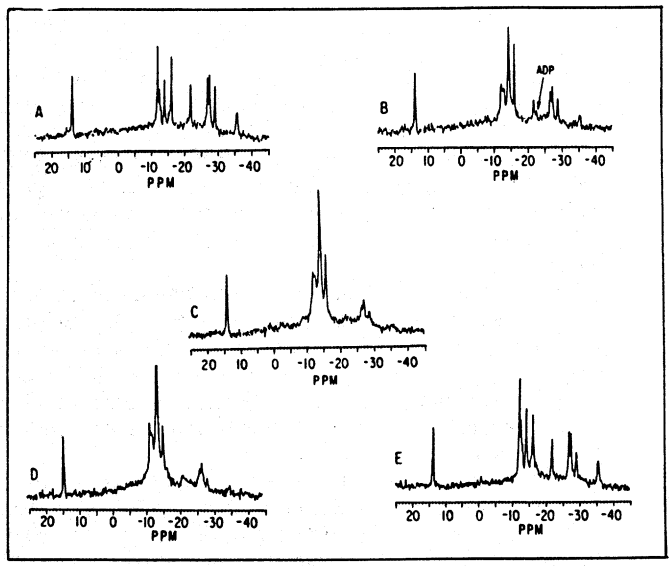

FIG. 8. A, ^{31}P spectrum (10,000 transients) taken as above perfused with a solution containing 0.1 mM $CaSO_4$, 50.0 mM glucose, 10.0 mM MES buffer (pH 6.0) O_2. B, Same as (A) except perfusate is saturated with N_2 54 to 81 min. C, Hypoxic state 189 to 216 min, treatment with perfusate containing 1.0 mM $MnCl_2$ for 108 to 135 min. D, Hypoxic state 297 to 324 min, after being washed out with non-Mn containing buffer for 81 to 108 min. E, Washout as in (D) with O_2 after 81 to 108 min.

of hypoxia in which the energy status of the cell is lowered (ADP levels are relatively high and anaerobic metabolism is dominant), minimal transport of Mn is observed as indicated by no observable line broadening with the exception of the resonances representing the already low concentration of ATP. We suggest that the small concentration of Mn^{2+} that is allowed to penetrate the plasma membrane is a result of the high (1.0 mM) concentration of Mn^{2+} on the outside of the cell, *i.e.* it is a consequence of full saturation of the energetically limited transporting 'apparatus.' The inability to affect the reverse migration of the low concentration of Mn^{2+} inside the cytoplasm under hypoxia indicates also that movement across the plasma membrane to the exterior of the cell is also an energy dependent process. Unlike the initial high concentration of Mn^{2+} outside the cell, the low Mn^{2+} concentration inside cannot saturate the limited number of energetic pathways to move the Mn^{2+} efficiently back out (Fig. 8D). Ultimately, the reintroduction of O_2 which restores the full energy status to the cells activates the system to move this small amount of trapped metal back across the plasma membrane (Fig. 8E).

It appears that the energy allotted for the uptake of divalent metal ions and possibly neutral molecules such as glucose (glucose-6-P is depleted during hypoxia even though the perfusate contains 50 mM glucose) can be effectively cut off when O_2-demanding energy transduction processes are inhibited. This energy may be in the form of a membrane potential. Thus the change in the internal pH of the cytoplasm from 7.6 to 7.0 could conceivably result in a potential which is insufficient to facilitate the membrane-bound metal ion carrier to move between the interior and exterior at a pH of 6.0.

More experiments are in progress to unravel the mechanism responsible for the movement of ions and uncharged molecules across both the plasma membrane and tonoplast.

SUMMARY

Excised corn root tips can be studied by ^{31}P NMR for extended periods of time under both neutral and acid conditions with little

change in intracellular pH and distribution of observable phosphate-containing compounds.

When stressed with Al^{3+}, the ^{31}P spectra of this root tissue shows a significant loss of ATP (45–65%) over a period of 20 h. Measured respiration rates also drop by 60 to 80% over this period of time. In the presence of 50 mM glucose, ATP levels remain high, as does respiration. The ^{31}P spectrum suggests that with the aid of glucose, Al^{3+} may be efficiently moved into the vacuole where it can no longer disturb metabolic processes in the cytoplasm.

Under aerobic conditions, the movement of paramagnetic manganese can be followed by the linebroadening of the ^{31}P spectra of the components in the cytoplasm and vacuole. Washing of the tissue exposed to Mn^{2+} removes the Mn^{2+} broadening from all resonances except the resonance representing phosphate in the vacuole, indicating final entrapment of Mn^{2+} in this compartment.

In a hypoxic state (N_2 atmosphere), the absence of O_2 restricts the energy available for the movement of Mn^{2+} into the cell as revealed by minimal ^{31}P linebroadening prior to washout. Introduction of an O_2 atmosphere restores the full sharp spectrum with no evidence of Mn^{2+} entrapment in the vacuole.

Thus, we conclude that in a state of diminished cellular metabolic activity (lack of O_2 or deficiency of glucose), the energy allotted to the facilitated movement of divalent ions such as Mn^{2+} is diverted to other processes in the cell, resulting in almost complete inhibition or slowing up of metal entry, respectively.

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